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(54) Title: LIPASE VARIANTS

(57) Abstract: Attaching a peptide extension to the C-terminal amino acid of a lipase re-duces the tendency to form odor. This may lead to lipase variants with a reduced odor generation when washing textile soiled with fat which includes relatively short-chain fatty acyl groups (e.g. up to C8) such as dairy stains containing butter fat or tropical oils such as coconut oil or palm kernel oil.

# LIPASE VARIANTS

## FIELD OF THE INVENTION

The present invention relates to lipase variants with reduced potential for odor generation and to a method of preparing them. It particularly relates to variants suited for use in detergent compositions, more particularly variants of the *Thermomyces lanuginosus* lipase showing a first-wash effect and a reduced tendency to form odors when washing cloth soiled with milk fat.

## BACKGROUND OF THE INVENTION

Lipases are useful, e.g., as detergent enzymes to remove lipid or fatty stains from clothes and other textiles, as additives to dough for bread and other baked products. Thus, a lipase derived from *Thermomyces lanuginosus* (synonym *Humicola lanuginosa*, EP 258 068 and EP 305 216) is sold for detergent use under the tradename Lipolase ® (product of Novo Nordisk A/S). WO 0060063 describes variants of the *T. lanuginosus* lipase with a particularly good first-wash performance in a detergent solution. WO 9704079, WO 9707202 and WO 0032758 also disclose variants of the *T. lanuginosus* lipase.

In some applications, it is of interest to minimize the formation of odor-generating short-chain fatty acids. Thus, it is known that laundry detergents with lipases may sometimes leave residual odors attached to cloth soiled with milk (EP 430315).

## SUMMARY OF THE INVENTION

The inventors have found that attaching a peptide extension to the C-terminal amino acid of a lipase may reduce the tendency to form odor. This may lead to lipase variants with a reduced odor generation when washing textile soiled with fat which includes relatively short-chain fatty acyl groups (e.g. up to $C_8$) such as dairy stains containing butter fat or tropical oils such as coconut oil or palm kernel oil. The variants may have an increased specificity for long-chain acyl groups over the short-chain acyl and/or an increased activity ratio at alkaline pH to neutral pH, i.e. a relatively low lipase activity at the neutral pH (around pH 7) during rinsing compared to the lipase activity at alkaline pH (e.g. pH 9 or 10) similar to the pH in a detergent solution.

Accordingly, the invention provides a method of producing a lipase by attaching a peptide extension to the C-terminal of a parent lipase and screening resulting polypeptides for lipases with any of the above improved properties.

The invention also provides a polypeptide having lipase activity and having an amino acid sequence which comprises a parent polypeptide with lipase activity and a peptide extension attached to the C-terminal of the parent polypeptide.

The invention further provides a detergent composition and a method of preparing a detergent using a lipase with the above properties.

## DETAILED DESCRIPTION OF THE INVENTION

### Parent lipase

The parent lipase may be a fungal lipase with an amino acid sequence having at least 50 % identity to the sequence of the *T. lanuginosus* lipase shown in SEQ ID NO: 2.

Thus, the parent lipase may be derived from a strain of *Talaromyces* or *Thermomyces*, particularly *Talaromyces thermophilus*, *Thermomyces ibadanensis*, *Talaromyces emersonii* or *Talaromyces byssochlamydoides*, using probes designed on the basis of the DNA sequences in this specification.

More particularly, the parent lipase may be a lipase isolated from the organisms indicated below and having the indicated amino acid sequence. Strains of *Escherichia coli* containing the genes were deposited under the terms of the Budapest Treaty with the DSMZ as follows:

| Source organism | Gene and polypeptide sequences | Clone deposit No. | Date deposited |
|---|---|---|---|
| *Thermomyces lanuginosus* DSM 4109 | SEQ ID NO: 1 and 2 | | |
| *Talaromyces thermophilus* ATCC 10518 | SEQ ID NO: 3 and 4 | DSM 14051 | 8 February 2001 |
| *Thermomyces ibadanensis* CBS 281.67 | SEQ ID NO: 5 and 6 | DSM 14049 | 8 February 2001 |
| *Talaromyces emersonii* UAMH 5005 | SEQ ID NO: 7 and 8 | DSM 14048 | 8 February 2001 |
| *Talaromyces byssochlamydoides* CBS 413.71 | SEQ ID NO: 9 and 10 | DSM 14047 | 8 February 2001 |

The above source organisms are freely available on commercial terms. The strain collections are at the following addresses:

DSMZ (Deutsche Sammlung von Microorganismen und Zellkulturen GmbH), Mascheroder Weg 1b, D-38124 Braunschweig DE

ATCC (American Type Culture Collection), 10801 University Boulevard, Manassas, VA 20110-2209, USA.

CBS (Centraalbureau voor Schimmelcultures), Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

UAMH (University of Alberta Mold Herbarium & Culture Collection), Devonian Botanic Garden, Edmonton, Alberta, Canada T6G 3GI.

Alternatively, the parent lipase may be a variant obtained by altering the amino acid sequence of any of the above lipases, particularly a variant having first-wash activity as described in WO 0060063 or as described below.

**Peptide extension at C-terminal**

The invention provides attachment of a peptide addition by a peptide bond to the C-terminal amino acid of a parent lipase (e.g. to L269 of the *T.* lanuginosus lipase shown as SEQ ID NO: 2). The peptide extension may be attached by site-directed or random mutagenesis.

The peptide extension at the C-terminal may consist of 2-15 amino acid residues, particularly 2-11 or 3-10, e.g. 2, 3, 4, 5, 7, 9 or 11 residues.

The extension may particularly have the following residues at the positions indicated (counting from the original C-terminal):

- a negative amino acid residue (e.g. D or E) at the first position,
- a small, electrically uncharged amino acid (e.g. S, T, V or L) at the 2nd and/or the 3rd position, and/or
- a positive amino acid residue (e.g. H or K) at the 3rd-7th position , particularly the 4th, 5th or 6th.

The peptide extension may be HTPSSGRGGHR or a truncated form thereof, e.g. HTPSSGRGG , HTPSSGR, HTPSS OR HTP. Other examples are KV, EST, LVY, RHT, SVF, SVT, TAD, TPA, AGVF and PGLPFKRV.

The peptide extension may be attached by mutagenesis using a vector (a plasmid) encoding the parent polypeptide and an oligonucleotide having a stop codon corresponding to an extension of 2-15 amino acids from the C-terminal. The nucleotides between the C-terminal and the stop codon may be random or may be biased to favor the amino acids described above. One way of doing this would be to design a DNA oligo, which contains the desired random mutations as well has the sequence necessary to hybridize to the 3´end of the gene of interest. This DNA oligo is used in a PCR reaction along with an oligo with the capability of hybridizing to the opposite DNA strand (as known to a person skilled in the art). The PCR fragment is then cloned into the desired context (expression vector).

**Increased long-chain/short-chain specificity**

The lipase of the invention may have an increased long-chain/short-chain specificity compared to the parent enzyme, e.g. an increased ratio of activity on long-chain (e.g. $C_{16}$-$C_{20}$) triglycerides to the activity on short-chain (e.g. $C_4$-$C_8$) triglycerides. This may be deter-

mined as the ratio of SLU with olive oil as the substrate and LU with tributyrin as substrate (methods described later in this specification).

**Increased alkaline/neutral activity ratio**

The lipase of the invention may have an increased alkaline/neutral activity ratio compared to the parent enzyme, i.e. an increased ratio of lipase activity (e.g. lipase activity) at alkaline pH (e.g. pH 9-10) to the activity at neutral pH (around pH 7). This may be determined with tributyrine as the substrate as described later in this specification.

**Substitution with positive amino acid**

The parent lipase may comprise one or more (e.g. 2-4, particularly two) substitutions of an electrically neutral or negatively charged amino acid with a positively charged amino acid near a position corresponding to E1 or Q249 of SEQ ID NO: 2. The positively charged amino acid may be K, R or H, particularly R. The negative or neutral amino acid may be any other amino acid,

The substitution is at the surface of the three-dimensional structure within 15 Å of E1 or Q249 of SEQ ID NO: 2, e.g. at a position corresponding to any of 1-11, 90, 95, 169, 171-175, 192-211, 213-226, 228-258 or 260-262.

The substitution may be within 10 Å of E1 or Q249, e.g. corresponding to any of positions 1-7, 10, 175, 195, 197-202, 204-206, 209, 215, 219-224, 230-239, 242-254.

The substitution may be within 15 Å of E1, e.g. corresponding to any of positions 1-11, 169, 171, 192-199, 217-225, 228-240, 243-247, 249, 261-262.

The substitution is most preferably within 10 Å of E1, e.g. corresponding to any of positions 1-7, 10, 219-224 and 230-239.

Thus, some particular substitutions are those corresponding to S3R, S224R, P229R, T231R, N233R, D234R and T244R.

**Amino acids at positions 90-101 and 210**

The parent lipase may particularly meet certain limitations on electrically charged amino acids at positions corresponding to 90-101 and 210. Lipases meeting the charge limitations are particularly effective in a detergent with high content of anionic.

Thus, amino acid 210 may be negative. E210 may be unchanged or it may have the substitution E210D/C/Y, particularly E210D.

The lipase may comprise a negatively charged amino acid at any of positions 90-101 (particularly 94-101), e.g. at position D96 and/or E99.

Further, the lipase may comprise a neutral or negative amino acid at position N94, i.e. N94(neutral or negative), e.g. N94N/D/E.

Also, the lipase may have a negative or neutral net electric charge in the region 90-101 (particularly 94-101), i.e. the number of negative amino acids may be equal to or greater than the number of positive amino acids. Thus, the region may be unchanged from Lipolase, having two negative amino acids (D96 and E99) and one positive (K98), and having a neutral amino acid at position 94 (N94), or the region may be modified by one or more substitutions.

Alternatively, two of the three amino acids N94, N96 and E99 may have a negative or unchanged electric charge. Thus, all three amino acids may be unchanged or may be changed by a conservative or negative substitution, i.e. N94(neutral or negative), D(negative) and E99(negative). Examples are N94D/E and D96E.

Further, one of the three amino acids N94, N96 and E99 may be substituted so as to increase the electric charge, i.e. N94(positive), D96(neutral or positive) or E99 (neutral or positive). Examples are N94K/R, D96I/L/N/S/W or E99N/Q/K/R/H.

The parent lipase may comprise a substitution corresponding to E99K combined with a negative amino acid in the region corresponding to 90-101, e.g. D96D/E.

The substitution of a neutral with a negative amino acid (N94D/E), may improve the performance in an anionic detergent. The substitution of a neutral amino acid with a positive amino acid (N94K/R) may provide a variant lipase with good performance both in an anionic detergent and in an anionic/non-ionic detergent (a detergent with e.g. 40-70 % anionic out of total surfactant).

**Amino acids at other positions**

The parent lipase may optionally comprise substitution of other amino acids, particularly less than 10 or less than 5 such substitutions. Examples are substitutions corresponding to Q249R/K/H, R209P/S and G91A in SEQ ID NO: 2. Further substitutions may, e.g., be made according to principles known in the art, e.g. substitutions described in WO 92/05249, WO 94/25577, WO 95/22615, WO 97/04079 and WO 97/07202.

**Parent lipase variants**

The parent lipase may comprise substitutions corresponding to G91G/A +E99E/D/R/K +T231T/S/R/K +N233N/Q/R/K +Q249Q/N/R/K in SEQ ID NO: 2. Some particular examples are variants with substitutions corresponding to the following.

| |
|---|
| T231R+ N233R |
| D96L+ T231R+ N233R |
| G91A+ E99K+ T231R+ N233R+ Q249R |
| R209P +T231R +N233R |
| E87K +G91D +D96L +G225P +T231R +N233R +Q249R +N251D |
| G91A +E99K +T189G +T231R +N233R +Q249R |

| |
|---|
| D102G +T231R +N233R +Q249R |
| N33Q +N94K +D96L +T231R +N233R +Q249R |
| N33Q +D96S +T231R +N233R +Q249R |
| N33Q +D96S +V228I + +T231R +N233R +Q249R |
| D62A +S83T + G91A +E99K +T231R +N233R +Q249R |
| E99N +N101S +T231R +N233R +Q249R |
| R84W +G91A +E99K +T231R +N233R +Q249R |
| V60G +D62E +G91A +E99K +T231R +N233R +Q249R |
| E99K +T231R +N233R +Q249R |
| T231R +N231R +Q249R |

**Nomenclature for amino acid modifications**

The nomenclature used herein for defining mutations is essentially as described in WO 92/05249. Thus, T231R indicates a substitution of T in position 231 with R.

270PGLPFKRV indicates a peptide extension attached to the C-terminal (L269) of SEQ ID NO: 2.

**Amino acid grouping**

In this specification, amino acids are classified as negatively charged, positively charged or electrically neutral according to their electric charge at pH 10, which is typical of detergents. Thus, negative amino acids are E, D, C (cysteine) and Y, particularly E and D. Positive amino acids are R, K and H, particularly R and K. Neutral amino acids are G, A, V, L, I, P, F, W, S, T, M, N, Q and C when forming part of a disulfide bridge. A substitution with another amino acid in the same group (negative, positive or neutral) is termed a conservative substitution.

The neutral amino acids may be divided into hydrophobic or non-polar (G, A, V, L, I, P, F, W and C as part of a disulfide bridge) and hydrophilic or polar (S, T, M, N, Q).

**Amino acid identity**

The parent lipase has an amino acid identity of at least 50 % with the *T. lanuginosus* lipase (SEQ ID NO: 2), particularly at least 55 %, at least 60 %, at least 75 %, at least 85 %, at least 90 %, more than 95 % or more than 98 %.

The degree of identity may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Jour-

nal of Molecular Biology, 48, 443-45), using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

**Amino acid sequence alignment**

In this specification, amino acid residues are identified by reference to SEQ ID NO: 2. To find corresponding positions in another lipase sequence, the sequence is aligned to SEQ ID NO: 2 by using the GAP alignment. GAP is provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45). The following settings are used for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

**DNA sequence, Expression vector, Host cell, Production of lipase**

The invention provides a DNA sequence encoding the lipase of the invention, an expression vector harboring the DNA sequence, and a transformed host cell containing the DNA sequence or the expression vector. These may be obtained by methods known in the art.

The invention also provides a method of producing the lipase by culturing the transformed host cell under conditions conducive for the production of the lipase and recovering the lipase from the resulting broth. The method may be practiced according to principles known in the art.

**Lipase activity**

Lipase activity on tributyrin at neutral and alkaline pH (LU7 and LU9)

A substrate for lipase is prepared by emulsifying tributyrin (glycerin tributyrate) using gum Arabic as emulsifier. The hydrolysis of tributyrin at 30 °C at pH 7 or 9 is followed in a pH-stat titration experiment. One unit of lipase activity (1 LU7 or 1 LU9) equals the amount of enzyme capable of releasing 1 µmol butyric acid/min at pH 7 or 9. LU7 is also referred to as LU.

The relative lipase activity at neutral and alkaline pH may be expressed as LU9/LU7. This ratio may be at least 2.0.

Lipase activity on triolein (SLU)

The lipase activity is measured at 30°C and pH 9 with a stabilized olive oil emulsion (Sigma catalog No. 800-1) as the substrate, in a 5 mM Tris buffer containing 40 mM NaCl and 5 mM calcium chloride. 2.5 ml of the substrate is mixed with 12.5 ml buffer, the pH is ad-

justed to 9, 0.5 ml of diluted lipase sample is added, and the amount of oleic acid formed is followed by titration with a pH stat.

One SLU is the amount of lipase which liberates 1 µmole of titratable oleic acid per minute under these conditions.

The lipase may particularly have an activity of at least 4000 or at least 5000 SLU/mg enzyme protein.

The relative activity towards long-chain and short-chain acyl bonds in triglycerides at alkaline pH may be expressed as the ratio of SLU to LU9. SLU/LU9 may be at least 2.0, at least 3.0 or at least 4.0.

**First-wash performance**

The first-wash performance of a lipase is determined as follows:

Style 400 cotton is cleaned by deionized water at 95°C and is cut in swatches of 9x9 cm. 50 µl of lard/Sudan red (0.75 mg dye/g of lard) is applied to the center of each swatch, and the soiled swatches are heat treated at 70°C for 25 minutes and cured overnight. 7 soiled swatches are washed for 20 minutes at 30°C in a Terg-O-Tometer test washing machine in 1000 ml of wash liquor with 4 g/L of test detergent in water with hardness of 15°dH ($Ca^{2+}/Mg^{2+}$ 4:1), followed by 15 minutes rinsing in tap water and drying overnight.

The lipase is added to the wash liquor at a dosage of 0.25 mg enzyme protein per liter. A control is made without addition of lipase variant.

The soil removal is evaluated by measuring the remission at 460 nm after the first washing cycle, and the results are expressed as ΔR by subtracting the remission of a blank washed at the same conditions without lipase.

**Test detergent**

The test detergent used in this specification has the following composition (in % by weight):

| | |
|---|---|
| Linear alkylbenzenesulfonate, $C_{10}$-$C_{13}$ | 12.6 |
| Alkyl sulfate, $C_{16}$-$C_{18}$ | 3.2 |
| Fatty acids, $C_{16}$-$C_{18, 18:2}$ | 0.9 |
| Alcohol ethoxylate, $C_{12}$-$C_{18}$, 6.7 EO | 13.2 |
| | |
| Zeolite | 35.2 |
| Sodium carbonate | 1.2 |
| Sodium hydrogencarbonate | 1.3 |
| Sodium silicate | 4.8 |
| Sodium sulfate | 1.9 |
| Sodium tetraborate | 2.7 |

| | |
|---|---|
| Phosphonate [1-hydroxyethane-1,2-diylbis(phosphonic acid)] | 0.1 |
| | |
| Sodium perborate monohydrate | 11.2 |
| Tetraacetylethylenediamine (TAED) | 6.3 |
| | |
| Copoly(acrylic acid/maleic acid) | 4.3 |
| SRP (soil release polymer) | 1.2 |

**Detergent additive**

According to the invention, the lipase may typically be used as an additive in a detergent composition. This additive is conveniently formulated as a non-dusting granulate, a stabilized liquid, a slurry or a protected enzyme. The additive may be prepared by methods known in the art.

**DETERGENT COMPOSITION**

The detergent compositions of the invention may for example, be formulated as hand and machine laundry detergent compositions including laundry additive compositions and compositions suitable for use in the pretreatment of stained fabrics, rinse added fabric softener compositions, and compositions for use in general household hard surface cleaning operations and dishwashing operations.

The detergent composition of the invention comprises the lipase of the invention and a surfactant. Additionally, it may optionally comprise a builder, another enzyme, a suds suppresser, a softening agent, a dye-transfer inhibiting agent and other components conventionally used in detergents such as soil-suspending agents, soil-releasing agents, optical brighteners, abrasives, bactericides, tarnish inhibitors, coloring agents, and/or encapsulated or non-encapsulated perfumes.

The detergent composition according to the invention can be in liquid, paste, gel, bar, tablet or granular forms. The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11, particularly 9-11. Granular compositions according to the present invention can also be in "compact form", i.e. they may have a relatively higher density than conventional granular detergents, i.e. form 550 to 950 g/l.

The lipase of the invention, or optionally another enzyme incorporated in the detergent composition, is normally incorporated in the detergent composition at a level from 0.00001% to 2% of enzyme protein by weight of the composition, preferably at a level from 0.0001% to 1% of enzyme protein by weight of the composition, more preferably at a level from 0.001% to 0.5% of enzyme protein by weight of the composition, even more preferably at a level from 0.01% to 0.2% of enzyme protein by weight of the composition.

The detergent composition of the invention may comprise the lipase in an amount corresponding to 1-5,000 LU per gram of detergent, preferably 2-500 LU/g, e.g. 10-100 LU/g. The detergent may be dissolved in water to produce a wash liquor containing lipase in an amount corresponding to 2.5-1,500 LU per liter of wash liquor, particularly 10 - 500 LU/l, e.g. 30-200 LU/l. The amount of lipase protein may be 0.001-10 mg per gram of detergent or 0.001-100 mg per liter of wash liquor.

The surfactant system may comprise nonionic, anionic, cationic, ampholytic, and/or zwitterionic surfactants. As described above, the lipase variants of the invention are particularly suited for detergents comprising a combination of anionic and nonionic surfactant with 70-100 % by weight of anionic surfactant and 0-30 % by weight of nonionic, particularly 80-100 % of anionic surfactant and 0-20 % nonionic. As further described, some preferred lipases of the invention are also suited for detergents comprising 40-70 % anionic and 30-60 % non-ionic surfactant. The surfactant is typically present at a level from 0.1% to 60% by weight, e.g. 1% to 40%, particularly 10-40 %. preferably from about 3% to about 20% by weight. Some examples of surfactants are described below.

Examples of anionic surfactants are alkyl sulfate, alkyl ethoxy sulfate, linear alkyl benzene sulfonate, alkyl alkoxylated sulfates.

Examples of anionic surfactants are polyalkylene oxide (e.g. polyethylene oxide) condensates of alkyl phenols, condensation products of primary and secondary aliphatic alcohols with ethylene oxide. polyethylene oxide condensates of alkyl phenols, condensation products of primary and secondary aliphatic alcohols, alkylpolysaccharides,and alkyl phenol ethoxylates and alcohol ethoxylates.

More specifically, the lipase of the invention may be incorporated in the detergent compositions described in WO 97/04079, WO 97/07202, WO 97/41212, WO 98/08939 and WO 97/43375.

## EXAMPLES

### Example 1: Preparation of lipase variants using C-terminal library

Creating the library:

The purpose was to add 3 extra amino acids to the C-terminal. Additional amino acids on the C-terminal could increase the activity towards long chained triglycerides as compared to short-chained triglycerides, as well as impede activity at pH7 as compared to activity at pH10, and thus diminish the smell attributed to the lipase in the detergent, during and after wash.

A plasmid pENi1576 was constructed with a gene encoding a lipase having the amino acid sequence shown in SEQ ID NO: 2 with the substitutions G91A+ E99K+ T231R+ N233R+ Q249R.

A PCR reaction was made using oligo19671 and 991222j1 (SEQ ID NO: 11 and 12) with pENi1576 as template in a total of 100 µl using PWO polymerase (Boehringer Mannheim). Oligo 991222J1 adds 3 extra amino acids on the C-terminal.

The PCR fragment was purified on a Biorad column and cut BamHI/SacII.

The plasmid pENI1861 (described in PCT/DK01/00805) was cut BamHI / SacII.

The PCR fragment and the plasmid vector was purified from a 1 % gel.

Vector and PCR fragment was ligated O/N, and electro-transformed into the *E.coli* strain DH10B giving 123,000 independent *E.coli* transformants.

10 independent clones were sequenced and showed satisfactory diversity.

A DNA-prep was made from all the clones.

*Aspergillus* transformation and screening.

Approximately 5 µg DNA plasmid was transformed into Jal355 (as mentioned in WO 00/24883). After 20 minutes incubation with PEG, the protoplasts were washed twice with 1.2 M sorbitol, 10 mM Tris pH7.5 (to remove $CaCl_2$).

The protoplasts were mixed in an alginate-solution (1.5 % alginate, 1 % dextran, 1.2 M sorbitol, 10 mM Tris pH 7.5). Using a pump (Ole Dich 110ACR.80G38.CH5A), this alginate solution dripped into a $CaCl_2$ – solution (1.2 M sorbitol, 10 mM Tris pH 7.5., 0.2 M $CaCl_2$ ) from a height of 15 cm. This created alginate beads of app. 2.5 mm in diameter with app. one transformed protoplast in every second bead. Approximately 55,000 transformants were generated.

After the beads had been made, they were transferred to 1.2 M sorbitol, 10 mM Tris pH7.5, 10 mM $CaCl_2$ and grown o/n at 30°C. The beads were washed twice with sterile water and afterwards transferred to 1*vogel (without a carbon source, which is already present in the alginate-beads (dextran)). The beads grew o/w at 30°C.

After o/w growth, the beads were spread on plates containing TIDE and olive oil (1 g/L agarose, 0.1 M Tris pH 9.0, 5 mM $CaCl_2$, 25 ml/L olive oil, 1.4 g/L TIDE, 0.004 % brilliant green). The plates were incubated o/n at 37°C.

384 positive beads were transferred to four 96 well microtiter plates containing 150 µl 1*vogel, 2 % maltose in each well.

The plates were grown for 3 days at 34°C.

Media was assayed for activity towards pnp-valerate and pnp-palmitate at pH7.5 (as described in WO 00/24883)). The 64 clones having the highest activity on the long-chained substrate (pnp-palmitate) as well as low activity on the short chained substrate (pnp-valerate) were isolated on small plates, from which they were inoculated into a 96 well microtiter plate containing 200 µl 1*vogel, 2 % maltose in each well.

After growth for 3 days at 34°C the media was once again assayed for activity towards pnp-valerate and pnp-palmitate at pH7.5 , as well as activity towards pnp-palmiate at pH10.

10 clones showed fine activity at pH10 towards pnp-palmitate and poor activity at pH7.5 towards pnp-valerate.

Due to a deletion in the DNA oligo, one variant accidentally had 11 amino acid residues extra on the C-terminal rather than 3.

Identified positive in first round:

G91A +E99K +T231R +N233R +Q249R +270SVT

G91A +E99K +T231R +N233R +Q249R +270TPA

G91A +E99K +T231R +N233R +Q249R +270SVF

G91A +E99K +T231R +N233R +Q249R +270HTPSSGRGGHR

The *Aspergillus* and screening procedure was repeated once again, thus identifying the following variants as positive:

G91A +E99K +T231R +N233R +Q249R +270LVY

G91A +E99K +T231R +N233R +Q249R +270EST

G91A +E99K +T231R +N233R +Q249R +270KV

G91A +E99K +T231R +N233R +Q249R +270RHT

G91A +E99K +T231R +N233R +Q249R +270TAD

**Example 2: Evaluation of odor and wash performance**

The following lipase variants based on SEQ ID NO: 2 were evaluated:

N94K +D96L +T231R +N233R +Q249R +270PGLPFKRV

G91A +E99K +T231R +N233R +Q249R + 270AGVF

G91A +E99K +T231R +N233R +Q249R +270HTPSSGRGGHR

G91A +E99K +T231R +N233R +Q249R +270HTPSSGRGG

G91A +E99K +T231R +N233R +Q249R +270HTPSSGR

G91A +E99K +T231R +N233R +Q249R +270HTPSS

G91A +E99K +T231R +N233R +Q249R +270HTP

G91A +E99K +T231R +N233R +Q249R +270SVF

G91A +E99K +T231R +N233R +Q249R +270LVY

G91A +E99K +T231R +N233R +Q249R +270EST

G91A +E99K +T231R +N233R +Q249R +270RHT

G91A +E99K +T231R +N233R +Q249R +270TAD

Washing tests were performed with cotton swatches soiled different soilings: lard/Sudan red and butter/Sudan red. The lard and butter swatches were heat treated at 70°C for 25 minutes and cured overnight. The soiled swatches were washed for 20 minutes at 30°C in a Terg-O-Tometer test washing machine in a wash liquor with 4 g/L of test detergent in water with hardness of 15°dH, followed by 15 minutes rinsing in tap water and drying overnight.

The lipase variant was added to the wash liquor at a dosage of 0.25 or 1.0 mg enzyme protein per liter. A control was made without addition of lipase variant, and a reference experiment was made with a lipase variant having the same amino acid sequence without any peptide extension.

The swatches were washed a second washing without lipase.

The performance was evaluated as follows:

- Odor generation was evaluated by a sensory panel, keeping the washed butter swatches in closed vials until the evaluation.
- Wash performance was evaluated by measuring the remission of the lard swatches after the first or the second washing. All variants showed a significant performance in this one-cycle washing test.
- A benefit/risk ratio was calculated as the performance on lard swatches after the first or second washing divided by the odor on butter swatches. An improved benefit/risk ratio indicates that the lipase can be dosed at a higher level than the reference to give wash performance on level with the reference with reduced odor.

All variants tested showed lower odor generation and/or a higher benefit/risk ratio than the same lipase without a peptide extension at the C-terminal.

**Example 3: First-wash performance, activity at alkaline/neutral pH, long-chain/short-chain activity**

The following lipase variants based on SEQ ID NO: 2 were evaluated:

G91A +E99K +T231R +N233R +Q249R +270HTPSSGRGGHR

G91A +E99K +T231R +N233R +Q249R +270HTPSSGRGG

G91A +E99K +T231R +N233R +Q249R +270HTPSSGR

G91A +E99K +T231R +N233R +Q249R +270HTPSS

G91A +E99K +T231R +N233R +Q249R +270EST

The first-wash performance was evaluated as described above, and each lipase variant was found to give a remission increase ($\Delta R$) above 3.0.

The lipase activity was determined as LU7, LU9 and SLU by the methods described above. Each lipase variant was found to have a LU9/LU7 ratio above 2.0 and a SLU/LU9 ratio above 2.0.

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| | | |
|---|---|---|
| 3 | The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on: | |
| 3-1 | page | 2 |
| 3-2 | line | 13-19 |
| 3-3 | Identification of Deposit | |
| 3-3-1 | Name of depositary institution | DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH |
| 3-3-2 | Address of depositary institution | Mascheroder Weg 1b, D-38124 Braunschweig, Germany |
| 3-3-3 | Date of deposit | 08 February 2001 (08.02.2001) |
| 3-3-4 | Accession Number | DSMZ 14049 |
| 3-4 | Additional Indications | NONE |
| 3-5 | Designated States for Which Indications are Made | all designated States |
| 3-6 | Separate Furnishing of Indications<br>These indications will be submitted to the International Bureau later | NONE |
| 4 | The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on: | |
| 4-1 | page | 2 |
| 4-2 | line | 13-19 |
| 4-3 | Identification of Deposit | |
| 4-3-1 | Name of depositary institution | DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH |
| 4-3-2 | Address of depositary institution | Mascheroder Weg 1b, D-38124 Braunschweig, Germany |
| 4-3-3 | Date of deposit | 08 February 2001 (08.02.2001) |
| 4-3-4 | Accession Number | DSMZ 14051 |
| 4-4 | Additional Indications | NONE |
| 4-5 | Designated States for Which Indications are Made | all designated States |
| 4-6 | Separate Furnishing of Indications<br>These indications will be submitted to the International Bureau later | NONE |

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## CLAIMS

1. A method of producing a polypeptide having lipase activity comprising:

a) preparing at least one polypeptide having an amino acid sequence which comprises:

i) a parent polypeptide having lipase activity and,

ii) a peptide extension attached to the C-terminal of the parent polypeptide,

b) selecting a polypeptide which has lipase activity and which compared to the parent polypeptide has:

i) a lower ratio between activities towards short-chain versus long-chain fatty acyl esters,

ii) a lower ratio between lipase activities at neutral versus alkaline pH, and/or

iii) a lower tendency to form odor in textile swatches with fatty soiling washed in detergent with the polypeptide,

c) producing the selected polypeptide.

2. The method of claim 1 wherein the parent polypeptide has an amino acid sequence which has at least 50 % identity with SEQ ID NO: 2.

3. The method of claim 1 or 2 wherein the peptide extension consists of 2-15 amino acid residues, particularly 3-10.

4. The method of any of claims 1-3 wherein the peptide extension comprises a positive amino acid residue at position 4, 5 or 6.

5. The method of any of claims 1-4 wherein the polypeptide is prepared by mutagenesis using of a plasmid encoding the parent polypeptide and an oligonucleotide having a stop codon corresponding to an extension of 2-15 amino acids.

6. A polypeptide having lipase activity and having an amino acid sequence which comprises:

a) a parent polypeptide having lipase activity and

b) a peptide extension comprising a positive, negative or polar amino acid residue attached to the C-terminal of the parent polypeptide.

7. The polypeptide of claim 6 wherein the parent polypeptide has an amino acid sequence which has at least 50 % identity with SEQ ID NO: 2.

8. The polypeptide of claim 6 or 7 wherein the parent polypeptide compared to SEQ ID NO: 2, comprises a substitution of an electrically neutral or negatively charged amino acid at the surface of the three-dimensional structure within 15 Å of E1 or Q249 with a positively charged amino acid.

9. The polypeptide of any of claims 6-8 wherein the parent polypeptide compared to SEQ ID NO: 2, comprises a substitution of an electrically neutral or negatively charged amino acid at a position corresponding to any of 1-11, 90, 95, 169, 171-175, 192-211, 213-226, 228-258 or 260-262.

10. The polypeptide of any of claims 6-9 wherein the parent polypeptide compared to SEQ ID NO: 2, comprises a substitution corresponding to E99K combined with a negative amino acid in the region corresponding to 90-101

11. The polypeptide of any of claims 6-10 wherein the parent polypeptide comprises a negative amino acid at a position corresponding to position E210 of SEQ ID NO: 2.

12. The polypeptide of any of claims 6-11 wherein the parent polypeptide comprises a negatively charged amino acid in the region corresponding to positions 90-101 of SEQ ID NO: 2.

13. The polypeptide of any of claims 6-12 wherein the parent polypeptide comprises a neutral or negative amino acid at a position corresponding to N94 of SEQ ID NO: 2 and/or has a negative or neutral net electric charge in the region corresponding to positions 90-101 of SEQ ID NO: 2.

14. The polypeptide of any of claims 6-13 wherein the peptide extension consists of 2-15 amino acid residues, particularly 3-10.

15. The polypeptide of any of claims 6-14 wherein the peptide extension comprises a positive amino acid residue at position 4, 5 or 6.

16. The polypeptide of any of claims 6-15 wherein the peptide extension is HTPSSGRGGHR or a truncated form thereof (particularly HTPSSGRGG, HTPSSGR, HTPSS or HTP), KV, EST, LVY, RHT, SVF, SVT, TAD, TPA, AGVF or PGLPFKRV.

17. A detergent composition comprising a surfactant and the polypeptide of any of claims 6-16.

18. A DNA sequence encoding the polypeptide of any of claims 6-16.

19. An expression vector harboring the DNA sequence of claim 18.

20. A transformed host cell containing the DNA sequence of claim 18 or the expression vector of claim 19.

21. A method of producing the polypeptide of any of claims 6-16 which method comprises culturing the transformed host cell of claim 7 under conditions conducive for the production of the polypeptide and recovering the polypeptide from the resulting broth.

22. A detergent composition comprising a surfactant and a lipase which has:

a) a remission increase (ΔR) of at least 3 at the test washing conditions given in the specification,

b) a ratio of hydrolytic activities towards tributyrin at pH 9 and pH 7 (LU9/LU7) of at least 2.0, and

c) a ratio of hydrolytic activities towards olive oil and tributyrin (SLU/LU) of at least 2.0.

23. A method of preparing a detergent, comprising:

a) testing at least one lipase for:

i) its first-wash performance in a detergent solution,

ii) its relative lipase activity at neutral and alkaline pH, and

iii) its relative activity towards long-chain and short-chain acyl bonds in triglycerides,

b) selecting a lipase which has:

i) a remission increase (ΔR) of at least 3 at the test washing conditions given in the specification,

ii) a ratio of hydrolytic activities towards tributyrin at pH 9 and pH 7 (LU9/LU7) of at least 2.0, and

iii) a ratio of hydrolytic activities towards olive oil and tributyrin (SLU/LU) of at least 2.0, and

c) mixing the selected lipase with a surfactant and optionally other detergent ingredients.